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M C Senut, … , S T Suhr, F H Gage

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Research Article

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Gene Transfer to the Rodent Placenta In Situ

A New Strategy for Delivering Gene Products to the Fetus

Marie-Claude Senut, Steven T. Suhr, and Fred H. Gage
Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, California 92037

Abstract

The delivery of biologically active factors to the developing mammalian embryo by in utero gene transfer has generated considerable interest but limited success. The chorioallantoic placenta is a potential alternative target for providing therapeutic transgenes to the fetus during gestation. We demonstrate that somatic gene transfer to the midgestation rat placenta may be efficiently accomplished in situ through the implantation of a variety of genetically modified cells with different antigenic and growth properties. Ex vivo–modified cells survived and retained transgene expression until term. Proteins secreted from the transplanted cells were detectable within the fetal trunk blood. These studies suggest that gene transfer to the placenta may be a useful tool for answering questions of both embryonic and placental development and providing therapeutic proteins during gestation for amelioration of diseases with onset during embryonic life. (*J. Clin. Invest.* 1998. 101:1565–1571.) Key words: gene transfer • placenta • transplantation • development • rat

Introduction

Many reports have emerged over the last several decades detailing mammalian development and the role played by the placenta in supporting fetal life. The placenta plays a critical role in many aspects of development of the mammalian embryo (1, 2) and is a highly complex organ consisting of connective tissue, vasculature, and specialized cell types (1). One unique feature of the placenta is its continued growth and differentiation during gestation. Trophoblast stem cells give rise to trophoblast giant cells, syncitiocytotrophoblasts, glycogen cells, and spongiotrophoblasts of the placenta (3, 4). In rodents and primates, invasive trophoblasts interact with uterine vessels and make contact directly with the maternal blood circulation (1). Although the mechanism of action is not yet well understood, trophoblast cells are involved in determination of the feto–maternal barrier and play a critical role in fetal development by transporting nutrients and gases from the mother to the fetus. Placental trophoblasts are also able to transfer a variety of biologically active substances such as antibodies (1, 5–7), nutrients (1), and trophic factors (8, 9) to the fetus.

Recent advances in the delivery of genes with disease-ameliorating properties to specific adult mammalian tissues have led to speculation that these techniques could also be applied to the developing embryo. Successful in utero gene transfer may also allow a better understanding of the role of specific factors during development and may ultimately provide new therapeutic approaches for diseases with pathologic onset during embryonic life which require prenatal correction of a defect. Over the last decade, a few studies have examined the potential of a variety of strategies to deliver genes of interest to the embryo. Fetal gene transfer has been attempted by the direct delivery of DNA vectors, including adenoviruses (10–12), retroviruses (13–15), and chemical vectors (16) through injections into the fetus (10, 11, 13–15), the amniotic cavity (10, 12), or the maternal blood (16). These reports suggest that fetal gene transfer is feasible; however, many of the methods tested have proven unpredictable or have interfered with fetal survival (15, 17).

An alternative approach to introducing biological factors to the developing embryo is to transfer novel genetic material to the chorioallantoic placenta. For many reasons, the placenta is an ideal host for gene transfer since it is present during the critical periods of embryonic development, and yet its involvement is transitory, lasting only as long as its association with the embryo. By targeting genes of interest to the placenta, biologically active proteins may be delivered to the developing embryo without direct modification of the fetal genome.

This investigation examines the proposition that factors secreted from genetically modified cells implanted to the placenta may be capable of entering the fetal blood supply. We show that autografts and, to a lesser extent, xenografts of genetically modified cultured cells survive and continue to produce detectable transgenes up to 6 d (term) after implantation into the midgestation rodent placenta. Furthermore, through the transplantation of human growth hormone (*hGH*)[1]-producing cells, we demonstrate that circulating levels of reporter protein can be detected in the fetal blood, indicating that the gene products secreted from the intraplacental grafts entered the developing embryo.

Methods

Primary cultures of rat placental cells

Primary cultures of rat placental cells were prepared as described previously (3). Briefly, pregnant Fischer 344 female rats on day 13 of

Marie-Claude Senut and Steven T. Suhr contributed equally to this work.

Address correspondence to Dr. Fred Gage, The Salk Institute for Biological Studies, Laboratory of Genetics, P.O. Box 85800, San Diego, CA 92186-5800. Phone: 619-453-4100 ext. 1012; FAX: 619-597-0824; E-mail: fred_gage@qm.salk.edu

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1. *Abbreviations used in this paper: βgal, β-galactosidase*; E, day of gestation; h293, human 293 cell line; *hGH*, human growth hormone.

gestation (E13) were anesthetized with an intramuscular injection of the following mixture: ketamine 44 mg/kg, acepromazine 0.75 mg/kg, and xylazine 4.0 mg/kg. The abdomen was shaved and wiped with Betadine and ethanol, a midline abdominal incision was made and both uterine horns were exposed. Embryos with their extraembryonic membranes and attached placentas were dissected from the uterus and placed in dishes containing HBSS. The petri dish was then transferred to a laminar flow hood, where, using fine forceps and iridectomy scissors, the placentas were carefully detached from the embryos and cleaned of overlying membranes, decidua, underlying yolk sac, and umbilical vessels. Using a scalpel and needles for microdissection, a tissue explant corresponding to the placental labyrinth was excised from each placenta, further washed in cold sterile HBSS, and transferred to petri dishes containing RPMI 1640 (Irvine Scientific, Santa Ana, CA) culture medium supplemented with 20% FBS (GIBCO, Grand Island, NY), 100 U/ml of gentamycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (4). Placentas were further dissected using fine forceps. Several explants were then placed in each culture dish of 6-well plates and maintained in the same placental medium at 37°C in a 5% CO_2 incubator as described previously (4). The medium was changed regularly and confluent cultures were passaged by trypsinization (ATV trypsin; Irvine Scientific), resuspended in placental medium, and plated. Cultured placenta-derived cells were designated RE131 (for rat E13, culture 1). Morphological, growth, and differentiation characteristics of RE131 cells were consistent with those described for other cultured rodent placental trophoblast precursor cells (3, 4).

Primary cultures of rat skin fibroblasts

The previously described primary rat skin fibroblast FF12 (18) was used in this study at passages 4–8 for retroviral infection.

Transgenes and retroviral vectors

The *LacZ* and *hGH* genes were inserted into retroviral vectors for production of infectious recombinant retroviruses (stable gene transfer) or used directly for transient transfection. Three expression constructs were used in the course of these experiments: *LgZnSN* (19), encoding the *Escherichia coli LacZ* gene fused to a nuclear localization signal and a selectable marker for neomycin resistance, and two constructs using a retroviral vector similar to the vector LINX-*vmyc* described by Hoshimaru et al. (20), except that the *v-myc* gene is replaced with *LacZ* (*L*-β-galactosidase [*βgal*]) (*Lβgal*) or *hGH* (*LhGH*).

In vitro gene transfer

Retroviral infection. *Lβgal* and *LhGH* plasmids were used to retrovirally infect fibroblasts, and *LgZnSN* plasmid was used for RE131 cells. Cells were plated at 10^5 (RE131) or 10^6 (fibroblasts) cells/100-mm plate and allowed to grow until 50–60% confluency. Recombinant retroviruses (~ 10^5 CFU/ml) were harvested from helper-free PA317 amphotropic packaging cells, filtered to remove cells and cellular debris, mixed with polybrene (10 μg/ml), and added to the plates of cells. The medium was changed 48 h after infection, and was replaced by G418-containing medium 1 wk later for selection. As colonies emerged after selection, individual clones were isolated and separately assessed for expression of *βgal* with histochemical (21) or immunohistochemical (see below) procedures, or tested for the highest *hGH* production as assessed by ELISA.

The *Lβgal* fibroblast line used in this report indicated 80% positive cell staining, and the highest expressing *LhGH* fibroblast clone selected produced 5–6 μg *hGH*/10^6 cells/d. No *hGH* was detected in *βgal*-positive or control fibroblasts. A helper-free *βgal*-expressing RE131 cell line isolated from the bulk population showed > 99% histochemical *βgal*-positive staining. Because primary rat placenta–derived cultured cells appeared to be resistant to infection with other retroviral vectors (M.-C. Senut, unpublished results), we were not successful in obtaining a stably expressing *hGH* placental cell line.

Calcium phosphate precipitation (22). *Lβgal* and *LhGH* plasmids were used to transiently transfect the human h293 cell line (h293) (23) 24–40 h before transplantation. Plasmid *pCH110* (Pharmacia, Piscataway, NJ) (24) was transfected at the same time as *LhGH* to allow determination of the transfection efficiency of duplicate plates by *βgal* staining. h293 cells were plated at 10^5 cells/100-mm petri dish and incubated until they reached 50% confluency. Cells were then incubated in a mixture of 500 μl 0.25 M $CaCl_2$, 500 μl 2× Hepes-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM $Na2HPO_4$-$2H_2O$, 12 mM dextrose, 50 mM Hepes), and 20 μg of plasmid. 2 d later, some cells were processed for histochemical staining (21) of *βgal* or ELISA-related determination of *hGH* production and the remaining cells were prepared for cell transplantation. *βgal* staining of the transfected cells revealed a transfer efficiency of ~ 50–60%. *hGH* levels in the culture medium from *LhGH* transfected cells indicated a maximum *hGH* secretion rate of 1.9 μg/10^6 cells/d at the time of transplantation. Control h293 cells did not secrete detectable *hGH*.

Preparation of cells for surgery and cell implantation

Timed pregnant Fischer 344 rats (E12–E15) were used in this study and were anesthetized with an intramuscular injection of the mixture described above. For grafting experiments, RE131 cells, fibroblasts, and h293 cells were grafted in the rat placentas as cell suspensions. After removal of the medium from the tissue culture flasks/plates, cells were washed, trypsinized, and counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Cells were centrifuged, pelleted, and resuspended at a concentration of 2–5 × 10^5 cells/μl in a grafting solution of Dulbecco's PBS. Cell suspensions were used within 2 h. All surgical procedures were performed in sterile conditions. After exposure of both uterine horns by a midline abdominal incision, the uterus was gently held by forceps and the embryos and their placentas were visualized by fiber optic illumination. Microliter quantities of cell suspension were injected through the uterine wall directly into each placenta using a 5-μl Hamilton microsyringe (Reno, NV). Control animals received no injections, vehicle injections, grafts of nonmodified cells, or grafts of *βgal* or *hGH*-positive cells. Using a 5-μl Hamilton syringe (30 gauge beveled needle), 2–5 μl of the cells were injected into each placenta of both uterine horns at a rate of 1 μl/6 s. In some placentas, several cell injections were performed. After the injections, the syringe was withdrawn slowly from the placentas and 5-0 sutures were used to carefully close the incision in the muscular abdominal wall of the dams. The skin was then closed with metal wound clips and the rats were allowed to recover from surgery. After surgery, nongrafted cells were put back into culture to verify their viability and *βgal* expression. Animals were analyzed at 2 d and 6 d after transplantation (Table I).

Table I. Gene Transfer in the Intact Placenta

Cells	Age at injection	Number of placentas	Survival time	Fetal survival
			d	%
RE131	E12–E14	31	6	33.5
RE131	E15	14	2	92.5
RE131	E15	8	6	88
FF	E15	12	2	75
FF-hGH	E15	13	2	69
FF	E15	30	6	80
FF-hGH	E15	45	6	82
h293	E15	6	2	100
h293-hGH	E15	34	2	94
h293	E15	13	6	69
h293-hGH	E15	26	6	85

Tissue preparation and immunohistochemistry

Animals were reanesthetized and the uterine horns were dissected out. The placentas were removed, fixed by immersion in either 4% paraformaldehyde or 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24–48 h, and cryoprotected in phosphate-buffered 30% sucrose. Coronal serial 40-μm-thick sections were cut on a freezing microtome and collected in 0.1 M Tris buffer. The sections were immediately processed for staining or kept at −20°C in cryoprotectant. Two series of sections were mounted on gelatin-coated slides for Nissl or hematoxylin/eosin stainings. A third series was immediately processed for histochemical detection of *βgal* as described previously (21). Immunohistochemistry was performed upon selected remaining series of sections. The primary mAbs directed against *E. coli βgal* (Promega, Madison, WI) and macrophages and monocytes (OX42; Serotec, Indianapolis, IN) were diluted at a final concentration of 1:5,000 and 1:500, respectively, in 0.1 M Tris-buffered saline containing 1% normal horse serum and 0.3% Triton X-100. Cultured cells or free floating sections were stained according to published procedures (25) using the avidin-biotin-peroxidase technique (Vectastain Elite; Vector Laboratories, Burlingame, CA) and diaminobenzidine.

hGH analysis by ELISA

At E17 and E21, control and *hGH*-grafted animals were anesthetized, the uterine horns were dissected out, and grafted concepti were explanted and segregated into the placenta and embryo for assessment of *hGH* protein levels. Other associated extraembryonic tissues were carefully removed. Placentas were dissected out and kept on ice until homogenization in ice-cold sample buffer. Placental homogenates were centrifuged and supernatants were used for *hGH* measurements. Blood samples were taken from the maternal tail vein and from the fetal trunk after decapitation. Blood samples were allowed to clot for several hours at 4°C and centrifuged at 4,000 rpm for 5 min for serum collection. Placental homogenates and blood samples were immediately processed for *hGH* quantification. *hGH* levels were measured by ELISA (range 0.0125–0.4 ng/ml; Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Absorbance of the ELISA samples was measured on a microplate reader (MR700; Dynatech Laboratories, Chantilly, VA) and compared to a standard curve generated using purified recombinant *hGH*. Homogenates and blood samples prepared from control animals were also processed to provide a baseline measurement.

Statistical analysis

The degree of statistical significance was determined by computer on the basis of Student's *t* test and ANOVA.

Results

Three different cell types were compared as donor cells for engraftment into the rat placenta: the rat placenta–derived RE131 cell line (see Methods), rat primary skin fibroblasts (18), and the h293 cell line (23). These cell types were chosen because they are amenable to gene transfer and exhibit different growth and antigenic properties. Two different transgenes were used in this study. The *LacZ* gene was used to histochemically label cells for the characterization and localization of grafts after intraplacental transplantation. The *hGH* gene was used for assay of protein migration from the placental transplant into the trunk blood of the embryo. *hGH* was chosen as a reporter gene, since secreted *hGH* levels can be detected sensitively and quantified using ELISA. In addition, previous experience with the *hGH* ELISA had indicated no significant cross-reaction with rat-derived tissue extracts or serum.

In situ transplantation of cultured cells into the rat placenta

To determine the feasibility of cell transplantation to introduce novel gene products into the placenta in situ, we examined the survival and transgene expression of genetically modified cell grafts implanted into the midgestation (E12–E15) placenta (Table I).

Intraplacental injection of cells was performed at E15 since a > 65% mortality rate of grafted embryos was observed when injections were performed at earlier times (Table I). In contrast, 69–100% of the fetuses with intraplacental grafts survived when injections were performed on or after E15. Grafted placentas and their associated embryos were indistinguishable in size and gross anatomical appearance from uninjected controls. To determine if cell transplantation affected placental growth, placental weights were monitored 6 d (E21) after transplantation of fibroblasts and h293 cells. No statistically significant differences in the placental weight were observed between grafted and control placentas (Fig. 1 *A*). Measurements of fetal weights performed 6 d after intraplacental implantation of primary fibroblasts indicated a slight decrease between control and fibroblast-grafted groups (4.475±0.085 vs. 3.977±0.103, $P = 0.022$) (Fig. 1 *B*); however, when assayed at 2 wk after birth, no statistically significant differences in weight was observed (Fig. 1 *C*). In some cases, pregnant rats were allowed to deliver and the neonates were observed for up to 2 mo after birth (66 d after injections). Neonates appeared healthy and exhibited normal growth and behavior compared to control animals.

Grafts of rat placenta–derived cells. Surviving RE131 cells were found in nearly 100% of the analyzed RE131-grafted placentas. Nissl staining of grafted placental sections showed a minimal disturbance of placental tissue organization (with the occasional exception of the needle track) (Fig. 2, *A* and *B*). Implanted placenta-derived cells could not easily be distinguished from the placental host cells on either Nissl or hematoxylin-eosin–stained sections. Histochemical detection of *βgal*-posi-

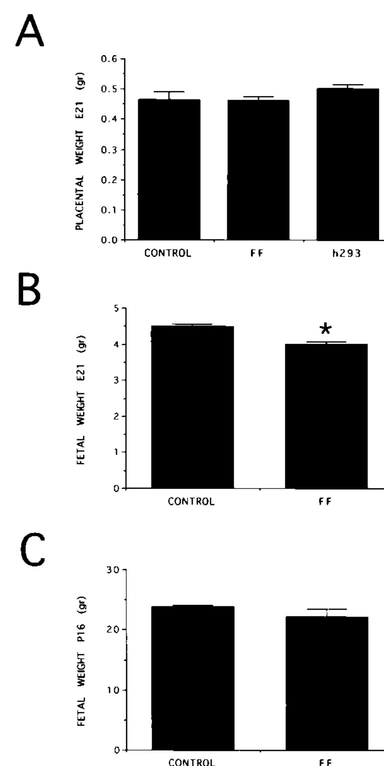

Figure 1. Effects of intraplacental cell transplantation on the weight of E21 placentas (*A*), E21 fetuses (*B*), and neonates at 16 d after birth (*P16*) (*C*). Values are mean±SEM. At all time points examined, the transplantation of genetically modified cells in the placenta did not alter the weight of placentas, fetuses, and neonates. *FF*, Primary rat fibroblasts.

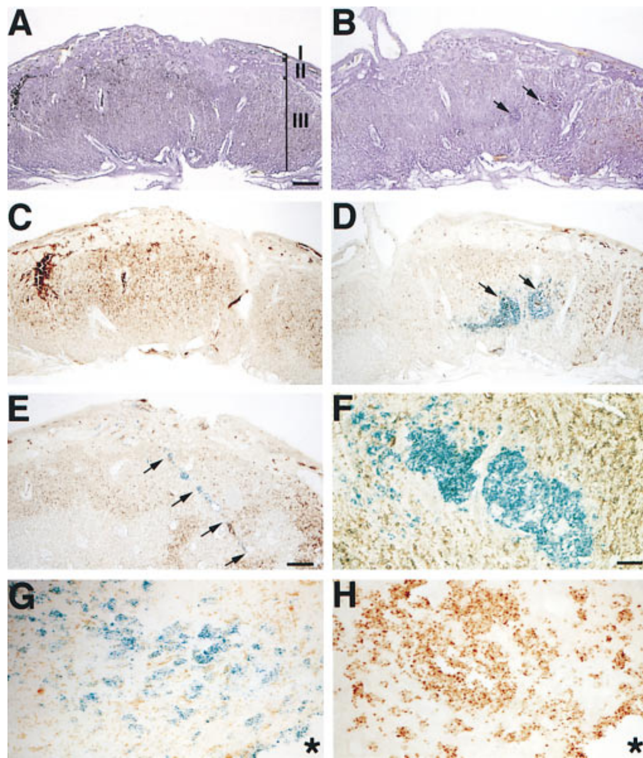

Figure 2. Bright-field microphotographs of frontal sections through control and RE131–grafted placentas. (*A*) Nissl staining illustrating the morphology of a control placenta at E17. *I*, Decidual zone; *II*, giant cell and basal cell zones; and *III*, labyrinth zone. (*B*) Nissl staining illustrating the morphology of a placental RE131 cell graft at E17. Even though the two injections sites (*arrows*) can be observed, the extent of RE131-B1 grafts is difficult to delineate. (*C*) Histochemical expression of *βgal* in a section successive to the section illustrated in *A*. (*D*) Histochemical expression of *βgal* in a section successive to the section illustrated in *B*. The arrows indicate the two injection sites. (*E*) Example of an RE131 cell graft at E17. Notice that the grafted cells are delineating the orientation of the injection (*arrows*). (*F*) Histochemical expression of *βgal* in an RE131 grafted placenta at E21 (6 d after grafting). (*G* and *H*) High magnifications of an RE131-B1 graft 2 d after grafting in the placenta (E17). Nuclear *βgal* expression was revealed in consecutive sections using histochemical (*G*) or immunohistochemical (*H*) detection. The asterisks locate the site of injection. Scale bar: 550 μm in *A*, *B*, *C*, and *D*; 600 μm in E; and 136 μm in *F*, *G*, and *H*.

tive RE131 cells revealed the presence of numerous positive grafted cells within the placentas at all time points examined, allowing for the determination of the size and boundaries of the intraplacental grafts (Fig. 2, *D–F*). Control placentas that received no injections and injections of vehicle or uninfected RE131 cells did not exhibit any detectable *βgal* staining (Fig. 2 *C*). As illustrated in Figs. 2 and 3, intraplacental grafts were primarily located in the placental labyrinth and sometimes occupied the more superficial giant cell and basal layers. In some cases, grafted cells concentrated along the injection track, delineating the orientation of the injection (Fig. 2 *E*). At both time points examined, the core of the grafts was primarily located at the injection sites (Fig. 2, *D* and *F*). Clusters of cells could be observed as much as 2.5 mm away from the presumptive injection site. Grafted primary cells were mixed with host placental cells and penetrated by blood vessels. The distribution patterns of staining were identical for both histochemical and immunohistochemical methods of *βgal* detection, further confirming the identity of the transplanted cells (Fig. 2, *G* and *H*). Few Nissl-stained inflammatory cells were observed within the RE131 grafts. This observation was confirmed by immunostaining of the macrophages and monocytes using the OX42 antibody (data not shown).

Grafts of primary rat fibroblasts. Like the placenta-derived RE131 cells, *βgal*-expressing and control primary rat fibroblasts survived transplantation into the placenta as assessed at E17 and E21. In contrast to RE131 cell grafts, however, the size and limits of the fibroblast grafts could be clearly delineated from the placental host cells after Nissl staining (Fig. 4, *A* and *B*). Grafted cells were found primarily in the labyrinth as described previously for RE131 cell grafts. Fibroblast grafts were frequently observed as well-defined cell aggregates composed of elongated and/or round-shaped cells with typical fibroblast morphology (Fig. 4 *C*). The cores of the grafts were primarily located at the presumptive injection sites (Fig. 4, *A* and *B*) and no migration of implanted cells was observed. In addition, blood vessels were observed within transplanted fibroblast grafts. There were no evident differences in graft size between E17 and E21. Fibroblast grafts extended over ~ 600 μm in the placenta and were surrounded and infiltrated by occasional inflammatory cells, including macrophages and/or monocytes as assessed by immunolabeling for OX42 (data not shown). Histochemical detection of *βgal*-positive fibroblasts and *βgal* staining revealed the presence of grafted cells within the placentas at all time points examined (Fig. 4 *D*). Control placentas that received injections of vehicle or control fibroblasts did not exhibit any detectable *βgal* staining.

Grafts of human 293 cells. h293 cells also survived implantation into the rat placenta at both time points examined. Grafted h293 cells could be unambiguously distinguished from the surrounding host tissue as assessed by Nissl staining (Fig. 4, *E* and *F*). h293 transplants were mostly found in the labyrinth layer and appeared as tightly arranged cell aggregates surrounding a lighter core composed of loosely arranged grafted cells mixed with inflammatory cells (Fig. 4 *E*). In some cases,

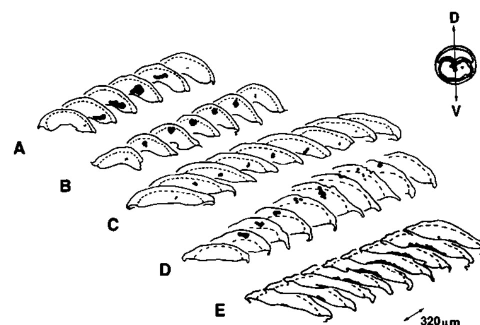

Figure 3. Schematic representation of "coronal" sections through RE131-B1 grafted placentas 2 d after implantation to illustrate the differences in graft extent and location. For each case, *A–E*, a series of sections, each separated by 320 μm, shows the distribution of grafted cells (*framed area*) in the placenta. The dotted lines delimit the inner labyrinth zone from the outer decidual, giant cell, and basal zones. The double arrow in the upper right gives the orientation of placental sectioning: *D*, dorsal; *V*, ventral.

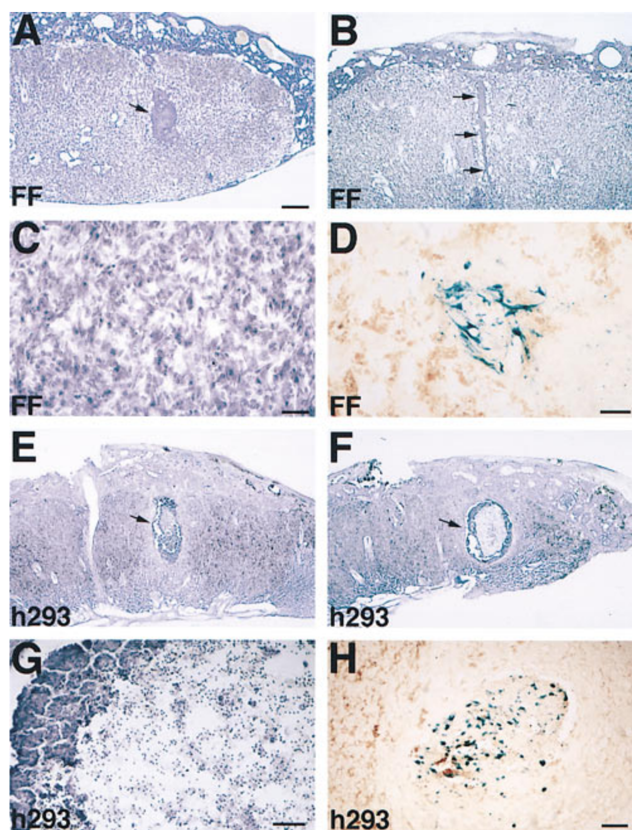

Figure 4. Bright-field microphotographs of frontal sections through primary fibroblast (*A–D*) or h293 cell (*E–H*) grafts. (*A* and *B*) Nissl staining illustrating the morphology of two different fibroblast grafts (*arrows*) at E21. In contrast to RE131 cell grafts, the limits of fibroblast grafts are easy to delineate. (*C*) High magnification of a Nissl-stained fibroblast graft at E21. (*D*) High magnification of a *βgal*-positive fibroblast graft at E21. (*E* and *F*) Nissl-staining illustrating the morphology of two different h293 cell grafts (*arrows*) at E17. (*G*) High magnification of the graft illustrated in (*F*) showing the large number of Nissl-stained inflammatory cells. (*H*) High magnification of a *βgal*-positive h293 cell graft 6 d after grafting in the placenta (E17). *FF*, Primary rat fibroblasts. Scale bar: 500 μm in *A*, *B*, *E*, and *F*; 45 μm in *C*; 50 μm in *D*; 41 μm in *G*; and 86 μm in *H*.

grafts displayed central necrotic cavities of various sizes (Fig. 4, *F* and *G*). In addition, few blood vessels were observed within the grafts. Like fibroblast grafts, h293 graft size did not change significantly between E17 and E21; however, the level of inflammatory response increased over time. Grafted cells distributed up to 1 mm away from the presumptive injection site. At E17 and E21, *βgal* staining revealed the presence of numerous *βgal*-positive h293 cells in the grafts (Fig. 4 *H*). Cells stained for *βgal* were mainly concentrated in the cell aggregates, although some positive h293 cells could also be found in the central inflammatory core.

Transplantation of hGH-producing cells in the rat placenta

To determine whether a factor secreted from the transplanted cells could be detected within the embryo, *hGH*-producing cells were transplanted into the rat placenta (Tables I and II) as described for the *LacZ* encoding cells. Experiments were carried out using both transiently transfected *hGH*-secreting h293 cells and retrovirally infected stable *hGH*-producing primary rat fibroblasts. *hGH* levels in both the placenta and fetal trunk serum were assessed at E17 and E21 (2 and 6 d after transplantation, respectively). At both time points examined, no *hGH* was detectable in the blood of dams with intraplacental transplants (data not shown).

Histological analysis indicated that *hGH*-secreting cell grafts survived intraplacental transplantation as well as *βgal*-producing grafts. Samples from uninjected placentas ($n = 23$) and from placentas implanted with control cells ($n = 44$) contained no detectable *hGH* as assessed by ELISA. In contrast, significant placental *hGH* levels were detected at both time points in animals that received *hGH*-secreting h293 cells and *hGH*-producing fibroblast grafts (Table II) (Fig. 5, *A* and *B*). To determine whether *hGH* secreted into the placental environment also entered the fetal blood, we determined *hGH* levels in blood harvested from the trunk of embryos at E17 and E21. No *hGH* was found in the sera of fetuses with uninjected placentas or with control cell-grafted placentas. In contrast, after intraplacental implantation of *hGH*-producing h293 cells or *hGH*-secreting rat fibroblasts, *hGH* levels could clearly be detected in the sera of the associated fetuses (Table II) (Fig. 5, *C* and *D*).

Discussion

The data presented here demonstrate that novel genes can be transferred and expressed in the rodent placenta using ex vivo gene transfer methods. Furthermore, we have shown that intraplacental transplantation of factor-secreting cells results in the expression of both detectable and measurable levels of protein in the fetal blood. To our knowledge, this is the first report examining cell engraftment to the rodent placenta and analyzing the transfer of foreign gene products from the transplant to the developing fetus.

Gene delivery during development has been primarily addressed through in vivo studies directed at examining gene transfer and expression in the mammalian embryo itself, after

Table II. Transplantation of hGH-producing Cells In the Rat Placenta (ELISA Studies)

Type of grafts	Age	Placentas injected	*hGH*-positive placentas	Mean *hGH* levels in placentas	*hGH*-positive fetuses	Mean *hGH* levels in fetuses
				pg/ml		*pg/ml*
hGH-h293	E17	34	24/34	199.39±36.18	9/24	66.76±15.78
hGH-h293	E21	26	13/26	255.39±57.30	3/13	18.08±6.27
hGH-FF	E17	9	9/9	1036.67±179.84	7/9	286.57±74.12
hGH-FF	E21	17	9/17	93.99±34.74	3/9	149.93±83.16

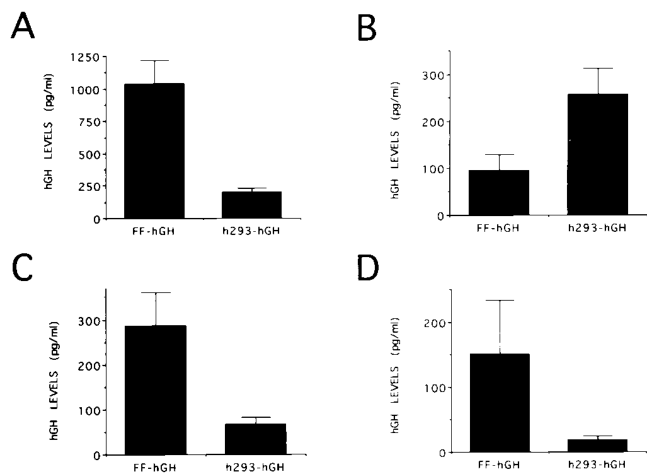

Figure 5. *hGH* levels in the placenta (*A* and *B*) and the fetus (trunk serum) (*C* and *D*) at 2 d (E17) (*A* and *C*) and 6 d (E21) (*B* and *D*) after transplantation of *hGH*-secreting fibroblasts and h293 cells. Values are mean±SEM.

injections of replication-defective retroviruses and adenoviruses into the fetus or the surrounding amniotic fluid (10–15). Although gene transfer and expression were achieved, those studies raised several issues associated with the use of adenoviral vectors, such as immune reaction, loss of transgene expression, potential mortality, and tissue targeting problems. In this study, we chose not to interfere directly with the fetus, but selected instead the placenta as the target for gene transfer. Although several studies have reported cell transplantation in the fetus (26), we are unaware of reports documenting the transplantation of genetically modified cultured cells into the placenta in situ. One reason that the placenta may not have been examined previously as a target for engraftment may be the impression that physical damage to the placenta (i.e., puncture by needles) is lethal to the developing embryo. We have found this to be true for placentas manipulated before E15; however, at later gestational ages, ≥ 70% fetal survival was observed even with multiple placental injections.

Since the mammalian placenta is considered immunotolerant, we examined the intraplacental survival and transgene expression of a variety of genetically modified cells with different antigenicity properties. The choice of autologously derived donor cells for ex vivo gene transfer was based on previous data demonstrating the improved in vivo survival of autologous grafts due to the reduced likelihood of immune responses (27). The observation that RE131 cells could not be clearly delineated on Nissl-stained sections suggests a favorable interaction with the host and that grafted RE131 cells become structurally integrated into the host tissue. We also examined whether primary cells of origin other than the placenta would be amenable to engraftment into the rat placenta. We chose primary rat skin fibroblasts because these cells can be readily obtained from skin biopsies, are easy to maintain, are amenable to retrovirus-mediated infection, and have been shown to survive well after implantation into heterologous locations including the central nervous system. Primary rat fibroblasts were also able to survive after intraplacental transplantation even though they did not seem to integrate into the host tissue as efficiently as the placenta-derived cells, as indicated by the clear delineation between grafted cells and host tissue. Although xenografts of *βgal*-producing h293 cells survived after intraplacental transplantation, they were associated with a strong cellular response from the host, characterized by inflammatory cell infiltrates not observed when RE131 cells or fibroblasts were transplanted. The origin of this inflammatory response (species difference and/or h293-released factor) has yet to be determined. These data corroborate recent studies documenting the possibility of immune responses after fetal viral injections (10, 11), and suggest that the placenta immune-privilege may not extend to highly antigenic transplants.

Histological studies led us to speculate that transgene products released from cells grafted into the placenta would be able to enter the fetal blood supply and the developing fetus. To test this hypothesis, we chose *hGH* as a reporter protein because it can be readily detected and quantified by ELISA. We have described the production of a nuclear-localized *βgal*-expressing RE131 cell line; however, *LacZ* gene transfer with the *LgZnSN* virus was only obtained after multiple trials of infection. Stable gene transfer using a variety of vectors and transgenes, including several vectors encoding *hGH*, was not successful (M.-C. Senut, unpublished data). Those findings suggest that RE131 cells may be resistant to infection by amphotropic or VSV-g protein pseudotyped Moloney murine leukemia virus–based retroviruses, and may require vectors of other origins for efficient infection. Throughout gestation, implanted *hGH*-secreting fibroblasts and h293 cells were able to express and deliver pharmacologic levels of *hGH* in the placenta, confirming the results obtained with *βgal* as a reporter gene. Persistence of detectable placental *hGH* levels until birth suggests that engrafted cells maintained their transgene expression throughout fetal development, given that the half-life of *hGH* is < 5 min in rodents (28). Experiments not described in this paper have shown that *hGH* or *βgal* positivity does not continue after parturition, suggesting that the *hGH* found in the fetal circulation is unlikely to result from cells translocalized from the placenta to the fetus. In none of 13 neonates which received intraplacental *hGH* grafts was *hGH* detectable in blood harvested 21 d after birth. Furthermore, we were unable to detect the presence of *βgal*-positive cells after histological examination of E21 concepti with intraplacental *βgal* grafts (data not shown). The decrease in placental *hGH* expression observed in fibroblast grafts at E21 might reflect either a decrease in gene expression, cell death, and/or a dilution of the *hGH* levels with the increasing volume of the sample. Indeed, rat placental volume increases by a factor of two between E17 and E21 (M.-C. Senut, unpublished observation). We were able to measure circulating *hGH* levels in the fetal serum up to 6 d after the transplantation of genetically modified primary fibroblasts and, to a lesser extent, after implantation of transfected h293 cells. The level of circulating fetal *hGH* was roughly proportional to the *hGH* cellular production rate. Thus, as compared to h293 cells, grafts of *hGH*-expressing fibroblasts which exhibited a higher *hGH* production rate in vitro also produced the highest circulating *hGH* levels after transplantation in the placenta. We expect that increasing the number of grafted cells and/or the rate of *hGH* production by the cells to be transplanted will further increase the level of circulating factor in the fetal blood. Although our study has not determined the precise mechanism of the *hGH* transfer, it sub-

stantiates the hypothesis that gene products produced in the placenta can cross over to the fetus.

This strategy for the delivery of gene products to the fetus has several potential applications. Engraftment of cells in the placenta can be used to further study placental development and the factors controlling placental cell proliferation, migration, and differentiation. Placental gene transfer could also be an approach to studying placental defects. Several diseases of the placenta are caused by defects in single genes controlling the proliferation or differentiation of placental trophoblasts, and gene transfer or transplantation studies can be examined as a means of rescuing the lethal phenotype (1). As we have demonstrated in this study, gene transfer to the placenta may also offer a unique opportunity to deliver biologically active substances to the fetus without direct gene transfer to either the mother or the fetus. For instance, transgenic mice with targeted neurotrophin deletions demonstrate various behavioral impairments associated with extensive loss in several neuronal populations and typically survive for less than a week after birth (29). The delivery of the missing neurotrophin via placental gene transfer could rescue affected neurons and promote survival after birth in such homozygous neurotrophin–null mutants. Ongoing studies in our laboratory are continuing to explore the potential of placental gene transfer techniques to deliver factors of interest to the developing embryo. It is our expectation that this gene transfer strategy will allow us to more fully define the role of various biologically active substances during mammalian development. It may ultimately provide a new therapeutic approach to the treatment of diseases with pathologic onset during early embryonic life.

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